29 *Emmonsia*

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CONTENTS

29.1 INTRODUCTION

29.1.1 Classification, Morphology, and Biology

The genus *Emmonsia* (obsolete synonym: *Haplosporangium*) belongs to the mitosporic Onygenales group, order Onygenales, class *Eurotiomycetes*, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi. The mitosporic Onygenales group consists of nine genera: *Blastomyces*, *Chrysosporium*, *Coccidioides*, *Emmonsia*, *Geomyces*, *Locazia*, *Malbranchea*, *Myriodontium*, and *Paracoccidioides*. Currently, the genus *Emmonsia* is divided into three recognized species, *Emmonsia crescens* (synonyms: *Emmonsia parva* var. *crescens* and *Chrysosporium parvum* var. *crescens*), *Emmonsia parva* (synonyms: *Emmonsia parva* var. *parva*, *Chrysosporium parvum*, and *Haplosporangium parvum*), and *Emmonsia pasteuriana*, and nine unassigned species [1]. The type species of the genus is *E. parva* [2].

Emmonsia crescens (an agent of adiaspiromycosis), *Blastomyces dermatitidis* (the agent of blastomycosis), and *Histoplasma capsulatum* (the agent of histoplasmosis) are known to form meiotic (sexual) stages, or teleomorphs, associated with the ascomycete genus *Ajellomyces* (Onygenaceae, Onygenales). However, no sexual stage is known for *Emmonsia parva* [3,4]. A recent phylogenetic study indicated that the genus *Emmonsia* forms part of a clade together with the genera *Ajellomyces*, *Blastomyces*, *Histoplasma*, and *Paracoccidioides* [2]. While *E. crescens* isolates are placed into two phylogenetic groups that correlate with their continents of origin (Eurasia and North America), *E. parva* isolates are more diverse: *B. dermatitidis* strains form a sister species of *E. parva* and *Histoplasma capsulatum* is also a close relative. *Paracoccidioides brasiliensis* and *Histoplasma capsulatum* are ancestral to most *Emmonsia* isolates [4].

Emmonsia grows moderately. Colonies are glabrous to velvety in texture, and are white, with buff to pale brown centers from the front, and cream to pale brown on the reverse. Hyphae are hyaline and septate; conidiophores are simple or occasionally branched at 90°C; unicellular, thin-walled, sub-spherical aleurioconidia (2–5 µm × 2–4 µm) are sessile or located on slender stalks, occurring in a solitary manner or in two- to three-celled chains. On blood or brain–heart infusion agar at 37°C–40°C and in vivo, thick-walled, large, liberated conidia are produced. These are adiaspores, which are clamydospore-like and nonreproducing.

E. crescens adiaspores are multinucleate (up to a few hundred nuclei), reaching diameter of 70 µm in vitro at 37°C and 700 µm in vivo; *Emmonsia parva* adiaspores are uninucleate, reaching diameters of 10–25 µm in vitro at 40°C and 40 µm in vivo. By contrast, *Emmonsia pasteuriana* does not produce adiaspores, but forms budding cell-like structures on brain–heart infusion agar at 37°C.

In infected lung tissues, inhaled conidia (2–4 µm) enlarge in the alveoli to become adiaconidia (up to 700 µm in diameter), which are surrounded by small granulomes, with concentric layers of fibrous tissue in later stages of the infection. Adiaspores do not replicate, and become calcified at the primary implantation site, leading to a minimal localized reaction in the host tissue. However, the regular pulmonary functions may be hampered by the presence of adiaspores.

Emmonsia is a cosmopolitan filamentous fungus that is present in soil and small mammals such as rodents that act as the reservoir host of the fungus. *Emmonsia* spp. are endemic in southwestern United States, Australia, and eastern Europe,

and are responsible for a pulmonary infection known as adiaspiromycosis in rodents, other small mammals, and occa-
AQ1 sionally in humans [6–9]. Human aspiromycosis typically occurs after inhalation of conidia of *Emmonsia*. While *Emmonsia crescens* is isolated primarily from humans, *Emmonsia parva* is often isolated from animals. *Emmonsia pasteuriana* was reported in an HIV-infected patient with a cutaneous disseminated infection.

29.1.2 Clinical Features and Epidemiology

Adiaspiromycosis is a lung disease of humans and small animals resulting from the inhalation of *Emmonsia* conidia, which elicit robust, multicellular immunologic response in tissue against the growing conidia, leading to noncaseating granulomas and disturbance of normal lung function. The first case of human adiaspiromycosis due to *Emmonsia* was described in France in 1964. Subsequently, cases of human pulmonary adiaspiromycosis have been reported in Russia, the Czech Republic, Brazil, Honduras, Guatemala, and the United States, with disseminated infection occurring in immunocompromised persons [10–21].

The establishment of adiaspiromycosis appears to be dose dependent, as a small inoculum often produces no or mild clinical symptoms and scattered radiological pathology, while a heavy or repeated inoculum may develop an acute, sometimes severe, pulmonary disease (often referred to as primary progressive pulmonary adiaspiromycosis, disseminated pulmonary adiaspiromycosis, or acute pulmonary adiaspiromycosis), with diffuse granulomatous lesions in both lungs. Cleaning or playing in closed environments inhabited only by bats and mice, where conidia of *Emmonsia* sp. are present in soil and dust, represents a risk factor for human adiaspiromycosis [22].

Although most human infections are attributable to *E. crescens*, a disseminated disease caused by *E. parva* in
AQ2 an AIDS patient has been described (Echaverria et al. 1993). *E. crescens* tends to form larger adiaspores and has a broader host range and geographic distribution than *E. parva*.

de Almeida Barbosa et al. [22] documented three Brazilian cases of acute pulmonary adiaspiromycosis due to *Emmonsia crescens*. The first case involved a 52-year-old man, who presented with cough, mucoid expectoration, fever, and weight loss over the previous 5 months. Chest roentgenogram showed diffuse micronodular infiltrates. Hematoxylin and eosin (H&E) staining of the transbronchial biopsy sections revealed helminth-like structures. Sections of lung biopsy revealed granulomas and characteristic adiaconidia as stained with fungal stains (periodic acid-Schiff [PAS] and
AQ3 Gomori-Grocott methamine-silver). The patient improved symptomatically without treatment. The second case concerned a 47-year-old man with nonproductive cough, dyspnea, fever, and weight loss. A chest roentgenogram revealed diffuse interstitial infiltrates in both lungs. H&E-stained sections of a transbronchial biopsy showed two granulomas, one of which contained an adiaconidium; sections stained by Gomori methenamine silver demonstrated a fungal structure of 100 μm in diameter. The patient recovered without treatment. The third case involved a 43-year-old man with a 1-month history of fever (39.5°C), cough with scant expectoration, weight loss (3 kg), dyspnea, and thoracic pain. A chest roentgenogram revealed bilateral micronodular infiltrates. H&E-stained sections of transbronchial biopsy demonstrated granulomas with characteristic adiaconidia. The patient had disrupted two mice nests, in a farm, 14 days before becoming ill. Treatment with itraconazole (200 mg/day) and prednisone (30 mg/day) alleviated patient's symptoms in a week, possibly reflecting the fact that enlargement of *Emmonsia* conidia causes a localized inflammatory response and *Emmonsia* conidia do not replicate and disseminate. AQ4

Nuorva et al. [23] described a case of disseminated bilateral pulmonary adiaspiromycosis in a 2-year-old Finnish girl. Electron microscopy showed that the three layers of the spore wall were not typical; rather, there seemed to be a gradual transition between the main wall zones, possibly split into thin layers. Varying numbers and thicknesses were seen with different staining methods and in different spores. This patient had become infected as a result of contact with soil dust containing the spores in the yard surrounding her home, and as a result of her mother's work in a large garden shop. She recovered from this rare infection after treatment with amphotericin B.

Dot et al. [24] presented a case of pulmonary adiaspiromycosis in a 30-year-old man with a 2-week history of unproductive cough, fever at 39°C, progressive dyspnea, thoracic pain, generalized weakness, and weight loss of 10 kg in 1 month. The patient became sick a few weeks after having played in the surroundings of an animal burrow, which may have functioned as a reservoir for *Emmonsia*. The chest radiography showed diffuse bilateral interstitial pneumonia with a micronodular pattern, and computerized tomography of the chest demonstrated disseminated pulmonary nodules. Formalin-fixed, paraffin-embedded transbronchial biopsy sections (4 μm) stained with PAS revealed round or ovoid, occasionally elliptical adiaspores (50–100 μm) with a thick wall in the center of some granulomas. Culture of the bronchoalveolar fluid (BAL) and transbronchial biopsy samples remained negative after 4 weeks. Sequencing analysis of PCR amplicon from BAL specimen using panfungal primers indicated *Emmonsia crescens* as the culprit. Treatment with oral itraconazole (200 mg/day) for 40 days improved his respiratory status and stabilized the pulmonary lesions.

Besides pulmonary adiaspiromycosis, Mendes et al. [25] described an outbreak of conjunctivitis involving 99 case-children in Brazil, with symptoms ranging from photophobia (57%), ocular pain (42%), to blurred vision (40%). Unlike conjunctivitis caused by bacterial or viral pathogens, neither purulent conjunctival discharge nor hemorrhage was observed, and family members of case-patient households were not commonly affected. Furthermore, the disease was characterized by unusual, single or multiple, white, opaque scleral nodules, often with hyperemia or local edema, and in some cases with opacification extending to the limbus, or angular corneal opacities and anterior uveitis with

granulomas in the anterior chamber. Microscopy of scleral biopsy samples identified spherical shapes, thick walls, and vacuous central area with lack of organized, internal structures consistent with adiaconidia of *Emmonsia* sp. (in contrast to *Coccidiodes immitis* spores that contain internal microsporules). Conjunctival irritation was likely due to exposure to *Emmonsia* conidia in dust caused in part by dry environmental conditions. Symptomatic children responded to corticosteroid treatment.

29.1.3 Diagnosis

The diagnosis of acute pulmonary adiaspiromycosis is based on the following criteria: (1) an acute onset of respiratory and systemic symptoms, (2) a radiological picture of diffuse micronodular lesions in both lungs, and (3) histopathological demonstration of the adiaconidia in the granulomas [26]. In particular, microscopic observation of large, round or oval, thick-walled adiaspores in histopathological sections of tissue using PAS or other stains provides a key diagnostic feature for adiaspiromycosis. Adiaspores are thick walled, enlarged conidia that may be uninucleate (*E. parva*) or multinucleate (*E. crescens*). Culture of *Emmonisia* spp. from sputum or BAL is not possible because adiaconidia do not multiply and remain trapped within lung granulomas.

Emmonisia spp. are differentiated from *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Paracoccidioides brasiliensis* by their inability to convert to a yeast phase at 37°C. They are differentiated from *Sporotrichum* by their inability to produce large chlamydospores at 25°C. *Emmonisia* spores in tissue (i.e., adiaspores or adiaconidia) resemble the parasitic spherules of *Coccidioides immitis* but differ by lacking internal spores.

The variable domain D2 located in the 5′ end of large subunit (LSU) nuclear rRNA contains sufficient nucleotide differences for discrimination between *Emmonisia* and its sibling *Blastomyces* species [27]. The other region of diagnostic interest is the internal transcribed spacer (ITS) region of rRNA (covering the ITS1, ITS2, and 5.8S rRNA), which offers a suitable target for examining *Emmonsia* species variability and for assessing evolutionary relationships among several related taxa.

29.2 METHODS

29.2.1 Sample Preparation

Biopsy specimens are stained with PAS stain and examined microscopically for adiaconidia-containing granulomas. Portions of the samples are cultured on Sabouraud agar with cycloheximide (0.5 mg/mL) at 30°C [24].

Emmonsia isolates are revived from either freeze dried or frozen (vapor phase of liquid nitrogen) stock and grown at 25°C on petri plates containing pablum cereal agar for 14–21 days. Blocks (1 cm × 1 cm) of mycelium and agar from cultures of *Emmonsia* species are excised from the culture plates and transferred to sterile snap-cap polypropylene tubes (12 mm × 75 mm; Fisher Scientific). The mycelial blocks are freeze dried using an Edwards Moduylo freeze-dryer [4].

Freeze-dried blocks of agar and *Emmonsia* mycelium (100 mg) are placed in 1.5 mL tubes and ground to a fine powder by using a 200-µL capacity pipettor tip. The fungal material is rehydrated with 500 µL of DNA extraction buffer (50 mM Tris, 10 mM EDTA, 1% sarcosyl, pH 8.0) with gentle agitation for 10 min. An equal volume of 1:1 chloroform–phenol is added to each tube, and mixed by shaking for 20 min. Then, the aqueous and organic phases are separated in a microcentrifuge for 5 min at 14,000 × *g*. The aqueous phase is pipetted into a clean tube, and 0.1 volume of 3 M sodium acetate pH 6.0 and 1.3 volumes of ethanol are added. The tube is sealed, and the contents are mixed by inverting the tube several times. Precipitated nucleic acids are pelleted by centrifugation at 14,000 × *g* for 1 min. Ethanol is decanted, and the pellet is dried by inverting the tube over an absorbent paper for 5 min. Nucleic acids are dissolved in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 250 µL of a saturated NaI solution and 10 µL of glass milk (GENECLEAN kit, Bio 101) are added to the tube. The tube is inverted periodically, and DNA is adsorbed onto the glass milk for 20 min. The glass milk is pelleted and rinsed, and the genomic DNA is eluted into 50 µL of 1/10-strength TE. DNA is stored at −20°C until used [4]. Alternatively, *Emmonsia* genomic DNA can be prepared by using Whatman FTA filter papers [28].

29.2.2 Detection Procedures

29.2.2.1 Sequencing Analysis of ITS Regions

Peterson and Sigler [4] used primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and D2R to amplify a 1200-nucleotide fragment including ITS1, ITS2, 5.8S rRNA, and part of the 28S rRNA for sequencing analysis of *Emmonsia* and related organisms.

Procedure

1. PCR mixture (100 µL) is composed of 1 µL of genomic DNA, 10 µL of 10× buffer, 1 µL of 50 µM primer ITS1, 1 µL of 50 µM primer D2R, 0.5 µL (2.5 U) of *Taq* polymerase, 10 µL of dNTP (1 mM concentration), and 76.5 µL of sterile deionized water.
2. Amplification is conducted with 30 cycles of 96°C for 30 s, 53°C for 30 s, and 72°C for 2.5 min followed by 10 min at 72°C. The amplified fragment is purified by using GENECLEAN kit (Bio 101) and eluted into 1/10-strength TE. The purified fragment is stored at −20°C until used in sequencing.
3. Sequencing is performed by using primers ITS1, ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), D1, D1R, D2, and D2R and Applied Biosystems DyeDeoxy sequencing kits. The sequencing reaction mixture is

prepared with 1.5 pmol of primer, 200–400 ng of the purified DNA fragment, and sequencing reagents. DNA sequences are analyzed on an ABI 373-automated DNA sequencer.

4. Alignment of overlapping and reverse complement strands to form a consensus sequence for each isolate is accomplished with an ASCII text editor. Alignment of sequences from different isolates is performed visually also by using an ASCII text editor. Phylogenetic relationships of isolates are determined by using PAUP 3.1.1. or by using programs from the PHYLIP package.

29.2.2.2 Panfungal PCR and Sequencing Analysis

Dot et al. [24] used panfungal primers His3 and His4 for PCR amplification and sequencing identification of *Emmonsia* and related genera. The primer His3 (5′-GTCGTAACAAGGTTTCCGTAG-3′) is located at the end of 18S rRNA at positions 9–29, and His4 (5′-AGCGGGTATCCCTACCTGAT-3′) is located at the beginning of 28S at positions 601–620 according in the sequence of *Histoplasma capsulatum* U18363. AQ6

Procedure

Genomic DNA is extracted from the BAL by using the High Pure PCR Template Preparation Kit (Roche Diagnostics).

1. PCR mixture (50 µL) is composed of 2 U of FastStart *Taq* DNA polymerase (Roche Diagnostics), 5 µL of 10× PCR buffer-$MgCl_2$ (20 mM), 20 pmol of each primer, 200 µM of dATP, dGTP, dTTP, and dCTP, and 5 µL of DNA.
2. Amplification is conducted in an iCycler IQ Thermal Cycler (Bio-Rad) with initial denaturation at 94°C for 3 min; 30 cycles at 94°C for 1 min, 58°C for 40 s, and 72°C for 1 min; and final extension at 72°C for 5 min.
3. PCR product (5 µL) is electrophoresed in a 2% agarose gel in the presence of ethidium bromide and visualized under UV light. The expected PCR product size is 613 bp. If the band is faint, reamplification of the product may be performed by using the same primers.
4. After purification, the PCR product is sequenced with the same PCR primers using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products are analyzed on an automated sequencer ABI Prism 3100 genetic analyzer. The BLAST analysis is used to compare the sequence with those at GenBank database.

29.2.2.3 Sequencing Analysis of LSU rRNA Gene

Untereiner et al. [2] utilized primers WNS9 and LR5 (5′-ATCCTGAGGGAAACTTC-3′) to amplify a DNA fragment that extends from the 3′ end of the nuclear small subunit rRNA gene to approximately 1000 bp positions downstream from the 5′ end of the nuclear LSU rRNA gene. The amplicon is purified using a QIAquick PCR Purification Kit (Qiagen). Sequencing reactions are performed using a Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and primers 5.8SR, LR1, WITS3, and WNS9. Excess dye terminators are removed by centrifugation using Centrisep columns (Princeton Separations) before analysis employing an Applied Biosystems 373A or 377 DNA sequencer.

Sequences are edited and assembled into larger consensus sequences using Sequencher 3.0 software (Gene Codes Corp.). Multiple alignments are produced using Clustal X version 1.7. The final multiple alignments are adjusted manually after visual inspection and areas of sequence ambiguity are eliminated. Phylogenetic relationships are inferred from aligned sequences using the maximum parsimony (MP) method found in PAUP (beta version 4.0b10). Gaps are treated as missing in all analyses. Heuristic searches are performed employing tree bisection–reconstruction (TBR) branch swapping with the MulTrees and steepest descent options activated, or using 1000 random addition sequence replicates.

29.3 CONCLUSION

Members of the genus *Emmonsia* are saprophytic fungi that are commonly present in soil and small mammals (e.g., rodents, otters, ground squirrels, goats, dogs, hedgehogs, raccoons, horses, and beavers). The teleomorphs of the genus *Emmonsia* along with the genera *Blastomyces* and *Histoplasma* are found in the genus *Ajellomyces.*

Among the species in the genus *Emmonsia*, *E. crescens* and *E. parva* spores are capable of entering the alveoli of mammalian hosts through inhalation, which expand to become adiaspores (adiacanidia), and cause a granulomatous inflammatory reaction, leading to a pulmonary disease known as adiaspiromycosis in animals and occasionally in humans. Most human adiaspiromycosis cases are due to *E. crescens*. Although human adiaspiromycosis often regresses spontaneously, sometimes the disease may persist, resulting in pneumonia or fatal respiratory failure [29,30].

Because *Emmonsia* spp. are not easily cultured, diagnosis of adiaspiromycosis has relied on histological examination of biopsies for large, round or oval, thick-walled adiaspores using PAS or other stains. Use of molecular techniques, especially sequencing analysis of the D2 region of the LSU rRNA gene and the ITS regions of rRNA biology, provides a valuable approach for improving the detection and diagnosis of human adiaspiromycosis.

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AUTHOR QUERIES

[AQ1] The term "man" has been changed to "humans" in the following sentences: "*Emmonsia* spp., Adiaspiromycosis is a lung disease…, and Among the species…" Please check and confirm the edit made.

[AQ2] Reference Echaverria et al. (1993) is not provided in the list. Please check.

[AQ3] Please check if "methamine" should be "metheanamine" in the term "Gomori-Grocott methamine-silver."

[AQ4] Please check the insertion of parenthesis to "200 mg/day" in the sentence starting "Treatment with itraconazole…"

[AQ5] Please check if "pippettor" should be "pipette" in the sentence "Freeze-dried…"

[AQ6] Please check the phrase "according in the sequence" in the sentence "The primer His3…" for sense.

[AQ7] Please provide accessed date for Ref. [1].

[AQ8] Please provide the in-text citation of Ref. [5].